Mycobiota associated with the rhodophyte alien speciesAsparagopsis taxiformis(Delile) Trevisan de Saint-Léon in the Mediterranean Sea

Mycobiota associated with the Rhodophyte alien species *Asparagopsis taxiformis* in the Mediterranean Sea

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Abstract
This study is the first to investigate and characterize the mycobiota associated with the alien species *Asparagopsis taxiformis*, a rhodophyte classified as one of the '100 Worst Invasive Species' in the Mediterranean Sea as it threatens biodiversity. Fungal endophyte and epiphyte communities were investigated on algal specimens from two sampling sites on the island of Linosa (MPA Isole Pelagie, Italy). 87% of the 24 specimens that were analysed for epiphytes displayed a microfungal colonization. No endophytes were found. Only a small amount of microfungi were found to be associated with this alga. Only five fungal *taxa* were isolated; two of which are sporadically associated to the alga, while three of which, *Eurotium rubrum*, *Cladosporium cladosporioides* and *C. pseudocladosporioides,* seem to be closely related to *A. taxiformis*. This scarcity could be related to algal chemical composition.

Keywords
Marine, fungi, red algae, seaweed

Introduction

The Rhodophyte *Asparagopsis taxiformis* is a dioecious, gametophytic alga that alternates its life cycle with a heteromorphic sporophyte known as *Falkenbergia hillebrandi* (Rojas *et al.* 1982). It mainly colonizes rocky bottom substrates and it is considered cosmopolitan in subtropical and tropical communities worldwide (Abbott 1999). The thalli of *A. taxiformis* are composed of sparsely branched, creeping stolons and erect shoots from which numerous side branches develop in all directions. They lack the hooked stolons that characterize the congeneric *Asparagopsis armata* (Andreakis *et al.* 2004). Even though it was first described near Alexandria, in Egypt (Delile 1813), recent studies have demonstrated that this alga could be considered to be an introduced species in the Mediterranean Sea. In fact, Andreakis and collaborators (2007) identified four lineages of *A. taxiformis* which behave as three biologically distinct but morphologically cryptic species (lineages 1 + 2, 3, and 4): lineages 1 and 4 are distributed in the Indo-Pacific Ocean, lineage 2 is found in the Indo-Pacific Ocean but is also present in the central Mediterranean Sea and southern Portugal, whereas lineage 3 is restricted to the Atlantic Ocean and to the Western coast of the Mediterranean Sea. Alien Western Mediterranean populations are rapidly expanding due to the absence of eco-physiological barriers to gene flow throughout their invasive trajectory (Andreakis *et al.* 2009). Since 2006, *A. taxiformis* has been classified as one of the '100 Worst Invasive Species' in the Mediterranean Sea (Streftaris & Zanetos 2006), as it threatens Mediterranean biodiversity.

It is well known nowadays that fungi in marine ecosystems play diverse ecological roles and have frequently been associated with parasitism of marine animals, plants, and algae (Richards *et al.* 2012). Algae are very numerous in marine habitats (9,200 to 12,500 described seaweeds) and cover vast areas of the sea bottom (Jones 2011). Algae-inhabiting fungi are known as algicolous and represent a taxonomically diverse group of mutualists, endosymbionts, parasites, pathogens and saprobes that are of evolutionary, ecological and economical interest (Zuccaro & Mitchell 2005). The ecological significance of host-microbe associations has hardly been investigated up to now. Fungi can live as epiphyites, but also as endophytes; in both cases they might excrete compounds that benefit their host, for example, in defense against pathogens, or to secure their living space inside the algal tissue (König *et al.* 2006). These interactions can sometimes lead to selective advantages for the algae, and it is particularly important to understand this phenomenon when we refer to alien species. It is well known that there are a large number of algae that are yet to be explored for the presence of fungi. According to Zuccaro & Mitchell (2005), red algae and brown seaweed seem to hold the greatest mycobiota diversity. As an example, the rhodophytes *Ballia* spp., *Laurencia* spp., *Palmaria palmata* and *Chondrus chrispus* exhibit saprophytic and parasitic fungal associations. These observations suggest that there is a huge mycobiota associated to red algae, but fungal biodiversity is yet to be explored (Shrearer *et al.* 2007).

The aim of this study is to investigate the epiphytic and the endophytic mycobiota associated with the invasive alga *A. taxiformis* in order to isolate and characterize these fungal strains. This will extend our knowledge of marine mycobiota and fungal-algae interactions, potentially providing us with tools that could be useful for the biocontrol of this invasive species.

Materials and methods

Study area - Samples of *A. taxiformis* were collected in July 2011 in Linosa, a small volcanic island in the Pelagian Archipelago (Lodola *et al.* 2012). This island is situated in a Marine Protected Area (MPA) located in the Central Mediterranean Sea (Sicily Channel - Italy). Despite the very low anthropogenic impact (i.e. no industries nor agriculture, and low tourism) and the low level of ship traffic and aquaculture, Linosa is recognized as being one of the main hotspots of the introduction of alien species in Italy (Occhipinti-Ambrogi *et al.* 2011). Two sampling sites were chosen in the area of maximum algal distribution: one in zone B, "Piscina" and one in zone C, "Secchitella" (Fig. 1). "Piscina" is a natural rocky pool located on the northeastern coast of the island that is connected to the open sea through a submerged tunnel. Samples were collected at an average depth of 3 meters. "Secchitella" is a relief of the sea bottom, placed about 500 meters off the southeastern coast of the island. Owing to its location in the open sea, the shoal is regularly exposed to strong marine currents (Lodola A., pers. obs.). Samples were collected at an average depth of 7 meters. A total of 32 gametophytes (16 for each sampling site), in perfect condition, were harvested and analysed by means of the following techniques.

Fungal isolation - The algal samples were collected in sterile containers to prevent contamination, and were maintained at about 4°C during transport. In the laboratory, the thalli were washed under running tap water to remove unrefined sediments and harvested in sterile containers. Under sterile conditions, on a laminar flow, the thalli were serially washed (three times) in seawater sterilized by autoclaving. As the main objective of this study was to identify the total mycobiota associated to *A. taxiformis*, different procedures were used to analyze both epiphytes and endophytes.

In order to isolate epiphytes, a total of 24 thalli (12 for each sampling site) were analyzed. Three different methods were applied, each using 8 thalli (4 per sampling site): 1) by means of sterile devices, approximately 0.05g fresh weight (0.001g dry weight) of each algal sample was homogenized in 500µl of sterile filtered seawater. 100µl of the homogenates were plated in sterile Petri dishes (60mm) containing 10ml of SWCMA medium (Sea Water Corn Meal Agar: 2g corn meal extract, 15g Agar dissolved in 1000ml of filtered seawater) (Panno *et al.* 2010, modified); 2) the thalli were divided into approximately three 1cm^2 pieces, each placed in wet chambers consisting of sterile Petri dishes (90mm) containing agarified seawater (SWA Sea Water Agar) (Kohlmeyer & Kohlmeyer 1979, modified) or 3) filtered sea water (SW) (Vrijmoed 2000, modified). For all three methods, an antibiotic mixture (70mgl^{-1} Penicillin, 200mgl^{-1} Streptomycin, 50mgl^{-1} Chloramphenicol), sterilized by filtration (0.2µm pores), was added to the sterile seawater to prevent bacterial growth, and 8 plates (corresponding to the algal thalli) were incubated at each selected temperature: 4°, 15°, 25°C.

In order to isolate endophytes, the following method suggested by Kjer *et al.* (2010) was used: samples were cut into small segments of approximately 1cm^2 and rinsed three times with sterile sea water to remove adherent surface debris; subsequently the pieces were immersed in EtOH 70% (vol/vol) for 60s for surface sterilization; they were then rinsed with sterile artificial sea water to stop the sterilization and carefully placed over the surface of a Petri dish containing the isolation medium A (15g malt extract, 24.4g artificial sea salt, 0.2g chloramphenicol, 15g agar, demineralized water up to 1000g, pH 7.4–7.8) (negative control); finally they were cut into smaller segments (approximately 0.2cm^2) with a sterile razor blade and placed on a second

Petri dish containing the same medium A. Four thalli were analyzed for each sampling site. Petri dishes were incubated at room temperature (20±5°C) in daylight. The plates were checked weekly for the first month, and then monthly for six months. When possible, the number of Colony Forming Units per gram of dry weight [CFU*(gdw)$^{-1}$] was recorded. Strains from each fungal morphotype from each sampling site were isolated in pure culture and deposited in the Pavia and Turin Mycoteques. Taxonomic identification of the isolated strains was carried out by means of both morphological and molecular approaches. Fungi were firstly identified morphologically on the basis of specific taxonomical keys (Raper & Fennel 1965; Sutton 1980; Bensch *et al.* 2012). Subsequently, molecular analyses were performed by sequencing ITS rDNA (White *et al.* 1990), LSU rDNA (Vilgalys & Hester 1990), and actin gene (ACT) (for *Cladosporium* spp., Carbone & Kohn 1999). Taxonomic assignments were based on phylogenetic position and similarity to reference sequences of the GenBank and CBS databases. Data related to the microfungal specimens isolated in this study were deposited in the NCBI database.

A phylogenetic tree was built according to Peláez and collaborators (2008). Alignment of the homologous regions for phylogenetic reconstruction was performed using the multiple alignment program ClustalW (Thompson *et al.* 1994). The 5.8S gene and the ITS2 internal transcribed spacer were used to infer phylogenetic relationships. Bayesian analysis (BY) based on Markov Monte Carlo chain approach was run as implemented in the computer program MrBayes 3.2 (Ronquist & Huelsenbeck 2012). To improve mixing of the chain, four incrementally heated simultaneous Monte Carlo Markov chains were run over 2,000,000 generations, using the GTR model of DNA substitution with gamma-distributed substitution rates. Trees were sampled every 100 generations, resulting in an overall sampling of 20,000 trees. The initial 1000 trees were not used for posterior analysis. A 50% majority-rule consensus tree was computed from the trees that were sampled after the process had reached stationarity, in order to get estimates for clade credibilities. The consensus tree was rooted using *Neurospora crassa* (Sordariaceae) as the outgroup (GenBank accession number M13906).

Results

A total of 32 algal thalli (8 per sampling site) were investigated. No fungus was isolated by the endophyte procedure. 87% of the 24 thalli that were analysed with the epiphyte methods displayed microfungal colonization. 5 fungal *taxa*, with different abundance in each sampling site were identified, as shown in table 1. *Eurotium rubrum* was found in all the analysed sites (B with methods 1 and 3, C with all methods), with a mean load of $1.27x10^4±3.08x10^3$CFU*(gdw)$^{-1}$. *Cladosporium pseudocladosporioides* was only isolated in thalli from zone B (all three methods, mean load: $5.7x10^4±1.9x10^4$CFU*(gdw)$^{-1}$), while *Cladosporium cladosporioides* was only isolated in thalli from zone C (all three methods, mean load: $1.27x10^4±3.08x10^3$ CFU*(gdw)$^{-1}$). *Alternaria* sp. and an unknown Xylariaceae were only found on two thalli in zone B with SW isolation procedure (15°C incubation temperature). Although the identification of *C. cladosporioides, C. pseudocladosporioides* and *Eurotium rubrum* was confirmed both morphologically and genetically, *Alternaria* sp. was only identified on the basis of molecular data, as it remains as sterile mycelium in axenic culture.

The Xylariaceae sp. strain (Fig. 2 a-f) remained unidentified. Morphologically, the isolate displays similar characteristics to the scolecosporus anamorphes of

Lopadostoma turgidum and *Nummularia* sp. (teleomorph: *Biscougnaxia*) (Xylariaceae), which were described as *Libertella*-like by Ju and collaborators (1993). However, discrepancy in microscopical features (i.e. conidia dimension, conidiophores structure) and the unavailability of teleomorph in pure culture did not allow us to assign it to a species. Comparison of ITS and LSU sequences with the available databases highlighted a high homology to Xylariaceae sequences, confirming morphological findings. However, there were insufficient homologies to allow us to assign the isolate to a specific genus. Moreover, high homologies (>98%) were found with sequences belonging to unidentified endophytes (Xylariales) of liverworts (Davis & Shwan 2008) and of *Viscum* sp. (Peršoh *et al.* 2010). Phylogenetic analyses were performed in order to understand the correct position of the isolate within the Xylariaceae family. Sequences representing 17 genus and 60 species were chosen from Xylariaceae taxonomic revision (Peláez *et al.* 2008). In addition, all available sequences from *Libertella* isolates as well as high homologous sequences (Fungal sp. and Fungal endophytes sequences) were included in the analyses. No sequences of *Lopadostoma* strains were found in the available molecular databases. Results are shown in figure 3. The unidentified Xylariaceae detected in this study, Fungal sp. and Fungal endophytes isolated from *Viscum* and from liverworts all form a defined cluster within the *Xylariaceae*. Sequences from *Nummularia* (*Biscougnaxia*) strains form distant clades.

Discussion

In recent years, many efforts have been made in the field of marine mycology to understand the composition, the structure and the function of fungal communities in marine environments. Nonetheless, data are still at the inventory stage and there are many regions and substrata that are yet to be explored.

Notwithstanding the large quantity of thalli analyzed in this study, very few epiphytic microfungi and no fungal endophytes seemed to be associated with *A. taxiformis.*

This scarcity can not be attributed to the isolation methods used in this study. On the contrary, the use of different techniques, associated with different incubation temperatures, undoubtedly maximized the number of possible isolates. In particular, the most effective methods, SWCMA and SW plates, allowed us to isolate not only fast growing strains, but also slow growing and peculiar strains like the unidentified *Xylariaceae* and *Alternaria* sp. (both only isolated with SW at 15°C incubation temperature).

Although our knowledge on fungal communities associated with Rhodophytes is still scarce and related to few species (Imhoff *et al.* 2006), the scanty mycobiota found to be associated with *A. taxiformis* seems to be compatible with other studies on microfungal communities of red algae. Only few fungal species (at most five) are reported from the Rhodophytes *Ceramimium rubrum*, *Chondrus crispus* and *Dumontia* sp. collected in British coastal waters, and *Rhodomela confervoides*, *Gelidium amansii*, *Gracilaria lemaneiformis*, *Ahnfeltiopsis flabelliformis* from the Fujiazhuang coastline of Dalian (Haythorn *et al.* 1980; Zang *et al.* 2009). Even in extreme environments, red algae seem to be poorly colonized: only two yeasts were isolated by Loque *et al.* (2010) from the Rhodophyte *Palmaria decipiens* in Antarctica, compared to the 63 and 10 fungal species isolated respectively from the brown seaweeds *Adenocystis utricularis* and *Desmarestia anceps* in the same environment.

To the best of our knowledge, this is the first study on mycobiota associated with a Rhodophyte in the Mediterranean Sea. Similarly to other investigated areas, there seems to be very little mycoflora associated with red algae compared to other substrates. As an example, Panno and collaborators found more than 70 (2013) and 90 (pers. obs) species associated with the marine macrophyte *Posidonia oceanica* and the green alga *Flabellia petiolata*. Such a small amount of fungi associated to red algae compared to other seaweeds may be correlated to the physiology of this Phylum. In fact, with more than 1500 compounds reported, the secondary metabolite chemistry of Rhodophyta is richer than those of other macroalgae, both in terms of abundance and diversity (Maschek & Baker 2008). What truly distinguishes red algae is that they are impressive producers of halogenated compounds, with over 90% of those reported containing bromine or chlorine, compared to only 7% of green algal compounds and less than 1% of those from brown algae (Harper *et al.* 2001). In particular, *A. taxiformis* releases a complex mixture of more than 120 halogenated metabolites containing less than five carbons in the longest chain (Paul & Pohnert 2011). It has been demonstrated that these compounds represent a selective advantage for the algae in a new environment, by altering the functional microbial flora of invertebrate neighbors (La Barre 2001). Moreover, extracts from *A. taxiformis* possess strong bioactivity against clinical pathogenic Gram-positive and negative bacteria (Ballantine *et al.* 1987; Val *et al.* 2001), spirochaete bacteria of clinical importance (Vedhagiri *et al.* 2009), fish and shellfish pathogenic bacteria (Genovese *et al.* 2012), shrimp *Vibrio* pathogens and plant pathogenic fungi (Manilal *et al.* 2009), algae (Rizvi & Shameel 2003), protozoa (Genovese *et al.* 2009) and nematodes (Rizvi & Shameel 2006), as well as antifouling properties (Manilal *et al.* 2010). Thus, microfungal strains that were found in this study have to bypass a wide range of chemical defenses in order to attach themselves to the substrate and to begin colonization. Indeed, the surface of macroalgae, despite providing nutrients, space and protection, can also create a barrier to fungal colonization by secreting growth-inhibiting metabolites into the phycosphere (Zuccaro & Mitchell 2005).

Overall, five *taxa* were identified, two of which seem to be sporadic.

Eurotium rubrum (anamorph *Aspergillus ruber*) seems to be closely related to *A. taxiformis*, as it was found, in differing abundance, in all the investigated areas and on the majority of the thalli (63%). Although *Eurotium rubrum* has already been endophitically isolated from the inner tissue of the mangrove plant (Dong-Li *et al.*, 2009) and from Dead Sea water (Kis-Papo *et al.* 2001; Butinar *et al.* 2005), it has never been detected as being associated with algae. Extracts of *A. taxiformis* have been demonstrated to be active against some *Aspergillus* species (*A. flavus*, *A. fumigatus*, *A. niger*) (del Val *et al.* 2001; Manilal *et al.* 2009). The *Eurotium rubrum* strain seems to be able to withstand these compounds. Moreover, in marine environments, isolates of this species have been found to produce potent radical scavenging compounds (Dong Li *et al.* 2009), as well as alkaloid and anthraquinone derivatives with antimicrobial and cytotoxic activity (Yan *et al.* 2012). Intriguing questions arise on the role of this fungus when associated with the Rhodophyte: does the fungus release compounds in *A. taxiformis* tissues? And if so, does this strain have a particular role in the association with this alga? Further investigations are required to clarify all the possible implications of this interaction.

Isolates from the *Cladosporium* genus were expected as this is one of the most frequently isolated *taxon* in marine environments (Jones *et al.* 2009; Panno *et al.* 2013). Marine *Cladosporium* strains seem to be interesting producers of secondary

metabolites in association with red algae, as in the case of *Porphyra yezoensis* (Ding *et al.* 2008). Two species, *C. cladosporioides* and *C. pseudocladosporioides*, were found in different sampling sites: *C. cladosporioides* seems to characterize thalli from zone C, while *C. pseudocladosporioides* seems to characterize thalli from zone B.
Differences observed in *Cladosporium* species composition could be related to multiple ecological and physicochemical factors. In particular, the two study sites differ in terms of exposure to hydrodynamic motions and to light (zone B can be considered a more protected environment than zone C). Thus, algae in a more exposed location (and with high nutrient rate due to high hydrodynamism as in the case of zone C) may grow faster and benefit from *extra* energy that can be used for the production of compounds against fungal attack. On the other hand, Rhodophytes can grow deeper than other algae, because they contain pigments that can absorb blue light waves. Blue light can also strongly affect fungal metabolism: it has been demonstrated that *C. cladosporioides* can grow twice as rapidly in response to blue light (Karpenko 2010), while for *C. pseudocladosporioides* this has not yet been examined. However, further investigations are required to confirm our findings.
A sterile isolate of *Alternaria* sp. was only obtained from one thallus. Identification of this strain at species level was not possible, as molecular tools alone are still not completely adequate methods of identification. Careful morphological identification of *in vitro* sporulating structure is still required for taxonomic characterization. Regarding the significance of this *taxon*, in literature *Alternaria* spp. are often reported in marine environments, but their role in marine ecosystems is still debatable (Jones *et al.* 2009).
The unidentified Xylariaceae strain isolated in this study is noteworthy. Neither morphological nor molecular analyses were able to allow us to assign the isolate to a known genus. Molecular analyses revealed its proximity to unidentified endophytes (Xylariales) of liverworts (Davis & Shwan 2008) and of *Viscum* sp. (Persoh *et al.* 2010). Nevertheless, in both studies, morphological identifications were not performed and taxonomical assignment was only based on molecular data. Phylogenetic analyses suggest a possible independence of the clade containing our isolates and of the Xylariales isolated by Davis and Shwan (2008) and by Persoh and collaborators (2010). Further investigations, including a comparison with *Lopadostoma* sequences, are required in order to confirm these findings.

Conclusion

The Rhodophyte *Asparagopsis taxiformis* represented a very interesting substrate to analyze for fungal association. Firstly, as far as we know, this is the first study to assess fungal mycobiota associated with this seaweed. Secondly, as this invasive species threatens Mediterranean biodiversity, knowledge on all aspects of its physiology and its ecology are of particular importance. Thirdly, *A. taxiformis* is an interesting substrate in terms of secondary metabolite chemistry. The role of natural products in the establishment and maintenance of the equilibrium between the host and the microbe or the ecological benefit of host/microbial metabolites are some of the most intriguing questions in marine fungal research.
Only three species, *Eurotium rubrum*, *Cladosporium cladosporioides* and *C. pseudocladosporioides*, seem to be closely related to *Asparagopsis taxiformis*. This Rhodophyte seems to be scarcely colonized by microfungi, and this may be due to the algal chemical composition. The *Asparagopsis taxiformis* specimens analyzed in this study all belong to lineage 2. This is considered to be an alien lineage in the

Mediterranean Sea, as it derives from an Indo-Pacific population that colonized the basin, most likely *via* the Suez Canal. One of the most important aspects of alien species introduction into a new environment is their relationship with microbiota. Lack of autochthonous associated microorganisms (parasites, mutalists, symbionts) in the new environment can result in differences in the establishment and in the spread of the new species. Thus, further studies on *A. taxiformis* lineages in their native environment may enhance the results of the present study in understanding the invasive processes of this species.

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Taxa		Presence/absence (number of colonized thalli) B zone "Piscina"	Presence/absence (number of colonized thalli) C zone "Secchitella"	CFU/(mg dw) ± ES (SWCMA)	% of colonyzed thalli (n. 24)	NCBI accession number	MUT accession number
PAV-M 1.133	Method 1	-	+ (4)	1.23 x 10^5 ± 6.6 x 10^4			
	Method 2	-	+ (2)				
Cladosporium cladosporioides	Method 3	-	+ (4)			KF415080	1417
(Fresen.) G.A. de Vries 1952	**Total**	**-**	**+ (10)**		42	KF415084	1384
PAV-M 1.134	Method 1	+ (4)	-	5.7 x 10^4 ± 1.9 x 10^4			
	Method 2	+ (1)	-				
Cladosporium pseudocladosporioides	Method 3	+ (2)	-			KF415078	
Bensch, Crous & U. Braun 2010	**Total**	**+ (7)**	**-**		30	KF415079	1396
PAV-M 1.4	Method 1	+ (4)	+ (4)	1.27 x 10^4 ± 3.08 x 10^3			
	Method 2	-	+ (4)				
Eurotium rubrum	Method 3	+ (1)	+ (2)				
W. Bremer 1901	**Total**	**+ (5)**	**+ (10)**		63	KF415077	1362
PAV-M 1.115	Method 1	-	-				
	Method 2	-	-				
	Method 3	+ (1)	-				
***Alternaria* sp.**	Total	**+ (1)**	**-**		0.04	KF415081	1366
PAV-M 1.116	Method 1	-	-				
	Method 2	-	-				
	Method 3	+ (1)	-			KF415082	
Unidentified Xilariaceae sp.	**Total**	**+ (1)**	**-**		0.04	KF415083	1369
% of colonyzed thalli on total sample (n.24)					**87**		

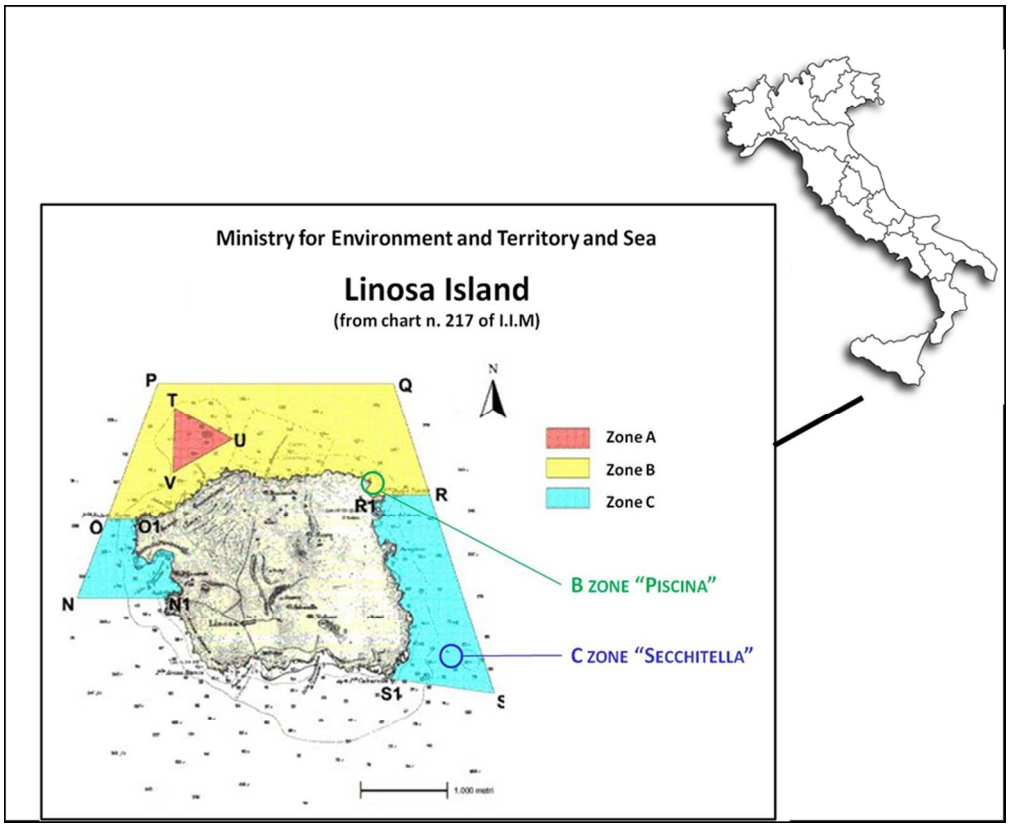

Fig. 1. Sampling sites of Asparagopsis taxiformis in Linosa Island. Capital letters stand for different level of protection in the Italian MPA: A means integral (strict), B general (multi-purpose) and C partial (buffer zone) reserve (Ministerial Decree, 2003) B and C zone are evidenced in green and blue, respectively. Modified from Area Marina Protetta Isole Pelagie www.isole-pelagie.it (Accessed 22 May 2013)

80x65mm (300 x 300 DPI)

Fig. 2 Unidentified Xylariales a) colony in axenic culture
74x60mm (300 x 300 DPI)

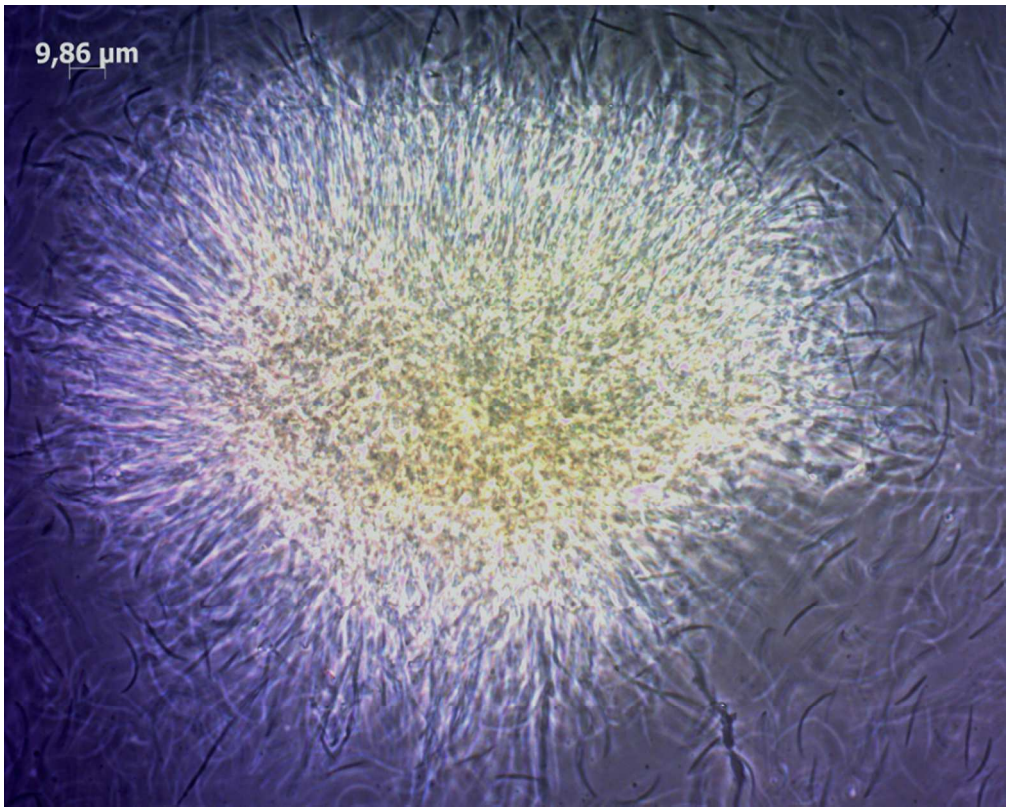

Fig. 2. Unidentified Xylariales b) a conidium-bearing region (200x);
75x60mm (300 x 300 DPI)

Fig. 2. Unidentified Xylariales c) Conidiophores (400x)
75x60mm (300 x 300 DPI)

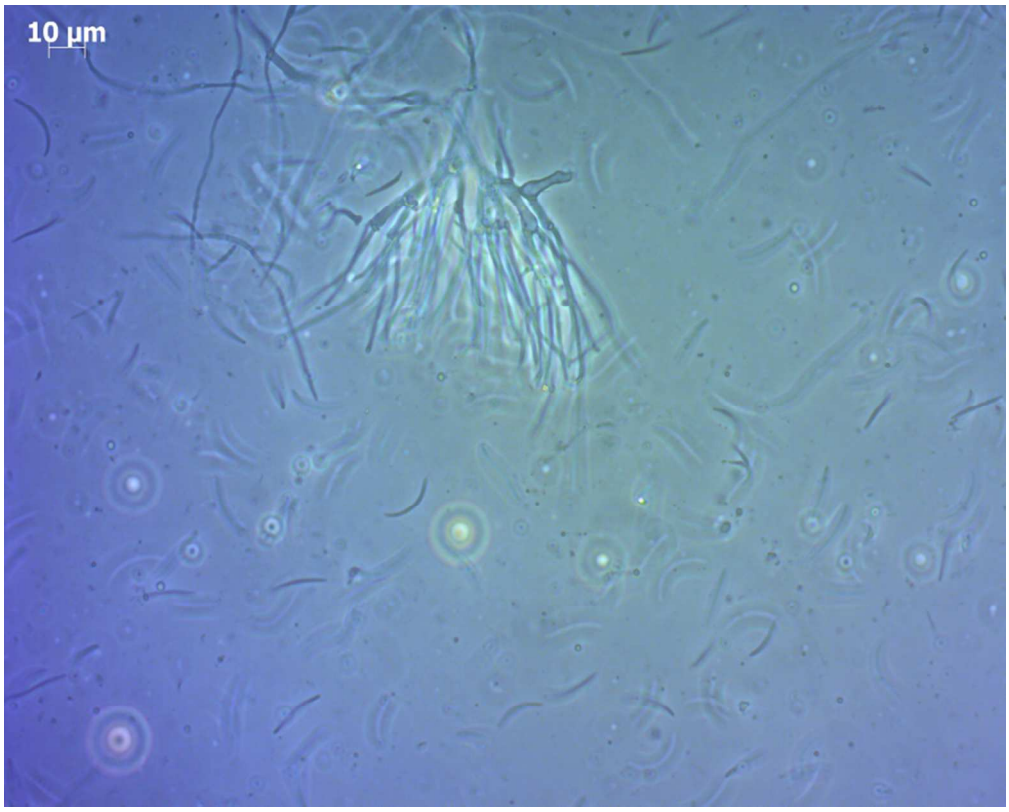

Fig. 2. Unidentified Xylariales d) Conidiophores (400x)
75x60mm (300 x 300 DPI)

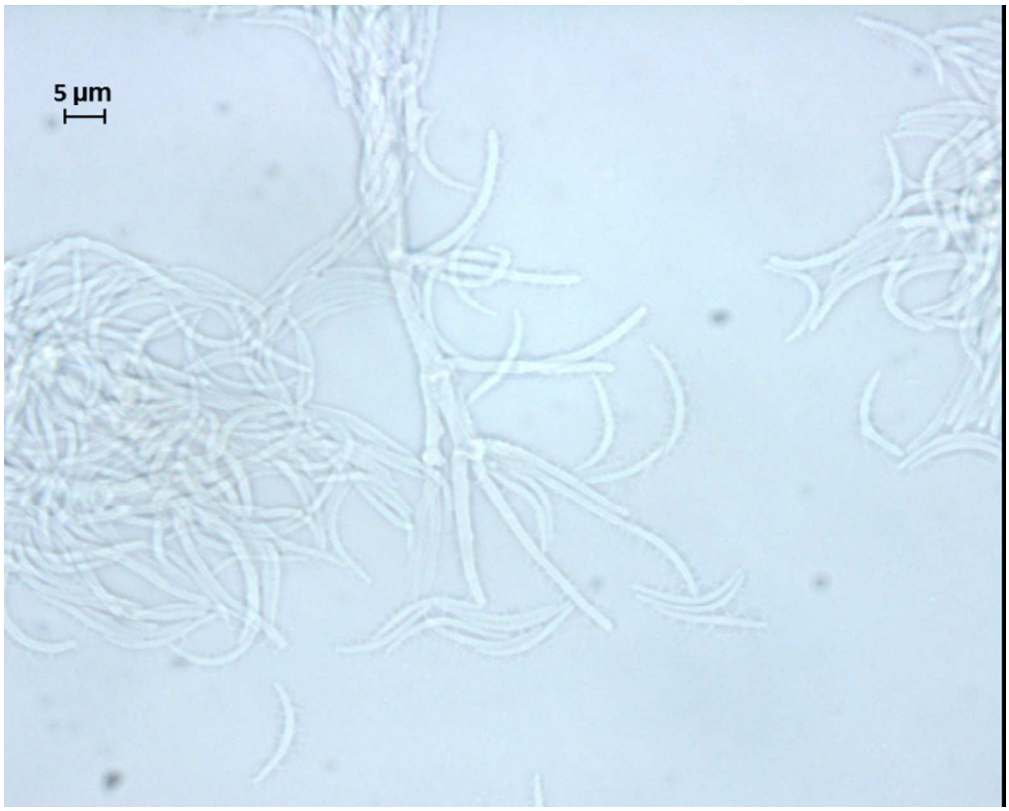

Fig. 2. Unidentified Xylariales e) Conidiophores (630x)
75x60mm (300 x 300 DPI)

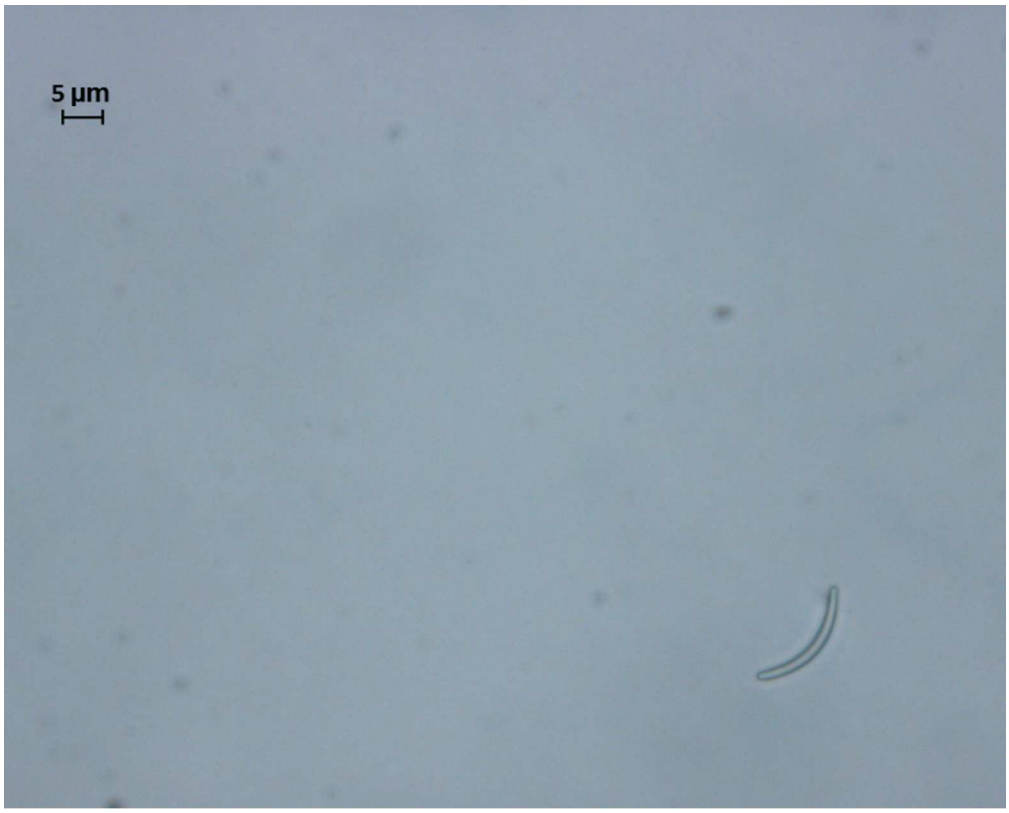

Fig. 2. Unidentified Xylariales f) Conidium (630x).
74x60mm (300 x 300 DPI)